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(54) Title: VECTOR FOR TRANSFECTION OF EUKARYOTIC CELLS

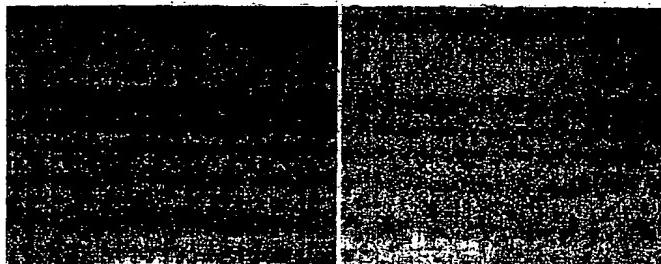
The Effects of VSVG on PLL-mediated Gene Transfer  
In Ht-1080 Cells and HepG2 Cells

A

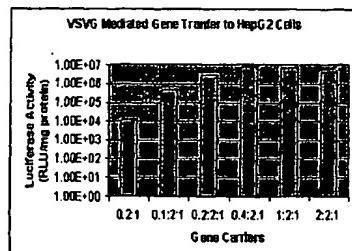
B

PLL-pDNA

VSVG-PLL-pDNA



C



(57) Abstract: Vectors comprising a nucleic acid, a nucleic acid binding polymer, a vesicle and a membrane active polypeptide are described. Preferred vectors facilitate transfection and/or reduce cytotoxicity. Methods of making the vectors and methods of using the vectors to transfect cells and/or treat a patient in need of gene therapy are described.

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**VECTOR FOR TRANSFECTION OF EUKARYOTIC CELLS****Field of the Invention**

The present invention relates to a vector comprising a cationic polymer that  
5 efficiently condenses nucleic acid and a lipid-based functional vesicle that carries a membrane active agent, such as viral envelope proteins or membrane active peptides which enhance efficiency of transfection of nucleic acids into eukaryotic cells with reduced cytotoxicity. The present invention also relates to methods of transfecting eukaryotic cells with such vectors.

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**Background of the Invention**

Gene therapy potentially offers a means of treating currently incurable genetic and acquired diseases (Verma, IM. Gene Therapy: Beyond 2000. Mol Ther. 2000 Jun.1(6):493). However, in this post-genomic era, the main problem with this therapeutic approach is a  
15 lack of effective gene delivery systems (Anderson, WF. Human Gene Therapy. Nature 392:25-30 (1998)). Gene delivery systems are designed to protect and control the location of a gene within the body by affecting the distribution and access of a gene expression system to the target cell, and/or recognition by a cell-surface receptor followed by intracellular trafficking and nuclear translocation (Friedmann, T. The Development of  
20 Human Gene Therapy. Cold Spring Harbor Laboratory Press. San Diego. 1999). Generally, there are two classes of gene vector systems: viral vector systems and non-viral vector systems. Viral vector systems include retroviral vector systems, lentiviral vector systems, adenoviral vector systems, adeno-associated viral vector systems, HSV viral vector systems, and alpha viral vector systems. Generally, viral vector systems have  
25 efficient gene transfer relative to non-viral gene carrier systems, because viruses have developed efficient mechanisms to overcome the gene transfer barrier in human beings. However, viral gene carrier systems have inherent disadvantages for use in the human body, such as the risk of wild-type virus regeneration, high immunogenicity and inflammation, and tumorigenesis (Verma IM, Stevenson J. Gene Therapy--Promises, Problems and  
30 Prospects. Nature. 1997 Sep 18;389(6648):239-42; Huang, L. and Viroonchatapan, E. Part I: Introduction in Nonviral Vectors for Gene Therapy. In L. Huang, M.C. Hung and E. Wanger (eds.). Nonviral Gene Vectors. Academic Press. (1999)).

The primary concern regarding gene carrier applications in medical gene therapy is safety and the potential of harm to cells. The severe limitations of viral vector systems greatly promote non-viral vector development. Synthetic non-viral gene carrier systems, include either naked plasmid DNA encoding therapeutic protein alone or with gene carriers such as a liposome-based lipoplex system, a polymer-based polyplex system or a lipid-polymer based lipopolyplex system (Felgner, PL., Zelphati, O., and Liang X. Advances in Synthetic Gene-delivery System Technology. In The Development of Human Gene Therapy. Friedmann, T (ed). Cold Spring Harbor Laboratory Press. San Diego. 1999). There are both similarities and difference between lipoplexes and polyplexes. From a physicochemical point of view, in both systems, DNA is incorporated into a complex as a result of bonds between cationic groups of lipids or polycations and anionic groups of the DNA. The driving force for such binding is the release of the low molecular mass counterions associated with the charged lipids or polymers into the external media, which is accompanied by a substantial entropy gain (Radler JO, Koltover I, Salditt T, Safinya CR. Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science*. 1997 Feb 7;275(5301):810-4). In the case of lipoplexes, the self-assembly process requires interaction between lipid molecules, as well as interaction with the DNA itself (Gershon H, Ghirlando R, Guttman SB, Minsky A. Mode of formation and structural features of DNA-cationic liposome complexes used for transfection. *Biochemistry*. 1993 Jul 20;32(28):7143-51). The resulting structure of the lipid molecules in the hydrophobic domain of the lipoplexes is one major factor that determines the macroscopic characteristics of such complexes, and in particular their size, shape, and stability in dispersion. In general, the ability to vary and control these parameters in lipid dispersions is relatively limited. Many lipoplexes are polydisperse and reveal strong non-equilibrium behavior involving variation in size, charge and stoichiometry (Lasic, DD. *Liposomes in Gene Delivery*, CRC Press, Boca Raton, FL, USA. 1997; Pouton CW, Lucas P, Thomas BJ, Uduehi AN, Milroy DA, Moss SH. Polycation-DNA complexes for gene delivery: a comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids. *J Control Release*. 1998 Apr 30;53(1-3):289-99; Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD, Pavlakis GN. Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nat Biotechnol*. 1997 Jul;15(7):647-52). In addition, the lipoplex systems

are often poorly water soluble and their macroscopic characteristics are unstable over time, limiting their pharmaceutical application.

Cationic polymer systems are similar to the cationic lipid systems in that they also have an overall positive charge and are capable of condensing plasmid DNA via ionic interactions (Kabanov, AV and Kabanov, AV. DNA complexes with polycations for the delivery of genetic material into cells. *Bioconjug Chem.* 1995 Jan-Feb;6(1):7-20). In contrast to lipid systems, the cationic polymer spontaneously forms complexes with DNA via electrostatic interactions. Self-assembly of polyplexes does not usually require interaction of the polycation molecules with each other. These positively charged DNA particles can efficiently bind to negatively charged cell membranes and thus can enhance DNA uptake by the cells, resulting in enhanced transfection efficiency. In such systems, a degree of flexibility can be achieved by varying the composition of the mixture (Pouton CW, Seymour LW. Key issues in non-viral gene delivery. *Adv. Drug Deliv. Rev.* 2001 Mar 1;46(1-3):187-203). Low immunogenicity typically allows polymers to be a biocompatible material for application in patients.

The cationic polymers commonly used as gene carrier backbones are poly(L-lysine) (PLL), polyethyleneimine (PEI), chitosan, dendrimers, and poly(2-dimethylamino)ethyl methacrylate (pDMAEMA).

Poly-L-lysine (PLL)-based polymers, pioneered in 1987, have been used for gene delivery by employing a targeting ligand, e.g. asialoorosomucoid, for transferring the gene and folate, to facilitate receptor-mediated uptake (Wu, GY., and Wu, CH. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. *J Biol Chem.* 1987 Apr 5;262(10):4429-32; Wu, GY., and Wu, CH. Receptor-mediated gene delivery and expression in vivo. *J Biol Chem.* 1988 Oct 15;263(29):14621-4; Mislick KA, Baldeschwieler JD, Kayyem JF, Meade TJ. Transfection of folate-polylysine DNA complexes: evidence for lysosomal delivery. *Bioconjug Chem.* 1995 Sep-Oct;6(5):512-5). PLL/DNA complexes are internalized into cells as a result of the interaction of a ligand displayed at the surface of the complex with the receptor (Wagner E, Zenke M, Cotten M, Beug H, Birnstiel ML. Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc Natl Acad Sci U S A.* 1990 May;87(9):3410-4). PLL-mediated gene transfer efficiency has been modified by employing lysosomaotropic agents (such as chloroquine) or inactivated adenovirus, or peptide derived from *Haemophilus influenzae* envelop proteins to

facilitate PLL/DNA complex release from the endosomes (Wagner E, Plank C, Zatloukal K, Cotten M, Birnstiel ML. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. Proc Natl Acad Sci U S A. 1992 Sep 1;89(17):7934-8; Curiel DT, Wagner E, Cotten M, Birnstiel ML, Agarwal S, Li CM, Loeschel S, Hu PC. High-efficiency gene transfer mediated by adenovirus coupled to DNA-polylysine complexes. Hum Gene Ther. 1992 Apr;3(2):147-54). Without the use of either targeting ligands or endosome lytic reagents, gene transfer is typically poor with PLL polyplexes alone, an important difference between the biological activity of the amphiphilic cationic lipids and the soluble polymer PLL.

Unlike PLL, both branched and linear polyethylenimine (PEI) show efficient gene transfer without the need for endosomolytic or targeting agents (Boussif O, Lezoualch F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A. 1995 Aug 1;92(16):7297-301). Positively charged PEI polyplexes are endocytosed by cells, and PEI is also believed to facilitate endosomal escape. Unfortunately, PEI has also been reported to be toxic to cells, which severely limits the potential for using PEI as a gene delivery tool in applications to human patients.

A range of polyamidoamine (PAMAM) dendrimers have been studied as gene-delivery systems (Eichman JD, Bielinska AU, Kukowska-Latallo JF, Baker JR Jr. The use of PAMAM dendrimers in the efficient transfer of genetic material into cells. Pharm. Sci. Technol. Today. 2000 Jul;3(7):232-245). Terminal amino groups bind DNA electrostatically, forming positively charged complexes, which are taken up by endocytosis. There are advantages associated with the star shape of the polymer as DNA appears to interact primarily with the surface primary amines, leaving the internal tertiary amines available to assist endosomal escape of the dendrimer-gene complex. Unfortunately, dendrimers have also been reported to be toxic to cells, a major limitation for its application in human patients.

Transfection efficiency and cytotoxicity are two of the most important factors that determine the yield of gene expression. All current cationic polymer gene delivery systems have drawbacks that hinder their use in gene therapies. The first drawback is that these systems are generally much less efficient in gene transfer experiments compared with viral

systems, especially in the case of PLL. The second drawback is that the cationic polymer gene carrier systems with higher gene transfer efficiency relative to PLL are usually toxic to the cells.

The natural process of viral infection is the transduction of foreign nucleic acids, the 5 viral genome, into host cells. The cytoplasmic membrane, endosome membrane and nuclear membrane are the three major intracellular barriers of virus transduction. To obtain high infectivity, some viruses have developed an envelope surrounding the virus, where envelope proteins are integrated in the envelope. Usually, viral envelope proteins have two functions: receptor binding and membrane fusion. Receptor binding facilitates transport of 10 the virus through the cell wall via receptor mediated endocytosis, and membrane fusion facilitates the escape of the virus from the endosome/lysosome, resulting in an increase in the number of transfected polymer/gene complexes transported into the nucleus.

Incorporation of viral elements into polymeric gene carrier systems is a strategy to enhance cationic gene carrier mediated gene transfer efficiency and reduce cytotoxicity. 15 Several viruses or viral envelope components have been used to modify lipid-mediated gene transfer in vitro and in vivo. UV inactivated whole defective Sendai virus (hemagglutinating virus of Japan, HVJ) has been used in lipid-based gene carrier systems to improve gene transfer in vitro and in vivo (Saeki Y, Matsumoto N, Nakano Y, Mori M, Awai K, Kaneda Y. Development and characterization of cationic liposomes conjugated 20 with HVJ (Sendai virus): reciprocal effect of cationic lipid for in vitro and in vivo gene transfer. Hum Gene Ther. 1997 Nov 20;8(17):2133-41). Curiel, D. T. et al. have reported that receptor-mediated transfection via transferrin-polylysine/DNA complexes is enhanced by simultaneously exposing the cells to defective adenovirus particles (Curiel DT, Agarwal S, Wagner E, Cotten M. Adenovirus enhancement of transferrin-polylysine-mediated gene 25 delivery. Proc Natl Acad Sci U S A. 1991 Oct 1;88(19):8850-4). These authors report that adenovirus particles function to disrupt endosomes containing the viral particle and the DNA complex. Replication-defective adenovirus particles and psoralen-inactivated adenovirus were reported to enhance transfection. Adenovirus enhancement of transfection is limited, however, to cells which have both a ligand receptor, e.g., transferrin receptor, 30 and an adenovirus receptor. Direct coupling of polylysine/DNA complexes to adenoviruses has also been employed for transfection (Curiel DT, Wagner E, Cotten M, Birnstiel ML, Agarwal S, Li CM, Loehel S, Hu PC. High-efficiency gene transfer mediated by

adenovirus coupled to DNA-polylysine complexes. *Hum Gene Ther.* 1992 Apr;3(2):147-54). Even though effective, the whole virus employed in the gene transfection system carries the risks of wild-type virus reformation and viral genome contamination. In related work, Wagner, E. et al. report augmentation of transfection in several cell lines when hemagglutinin HA-2 N-terminal fusogenic peptides from influenza virus are included in transferrin-polylysine-DNA complexes (Wagner E, Plank C, Zatloukal K, Cotten M, Birnstiel ML. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene transfer vehicle. *Proc Natl Acad Sci U S A.* 1992 Sep 1;89(17):7934-8). Vesicular stomatitis virus G envelope protein (VSVG) has also been reported to help lipid-DNA complex in vitro gene transfer (Abe A, Chen ST, Miyanohara A, Friedmann T. In vitro cell-free conversion of noninfectious Moloney retrovirus particles to an infectious form by the addition of the vesicular stomatitis virus surrogate envelope G protein. *J Virol.* 1998 Aug;72(8):6356-61; Abe A, Miyanohara A, Friedmann T Enhanced gene transfer with fusogenic liposomes containing vesicular stomatitis virus G glycoprotein. *J Virol.* 1998 Jul;72(7):6159-63).

The glycoprotein (VSVG) derived from the vesicular stomatitis virus, a member of the rhabdovirus family, is a viral envelope protein that has been widely used in pseudotyping viral vectors to improve gene transduction efficiency. VSVG is a transmembrane protein and induces membrane fusion at acidic pH in the absence of other viral components (Florkiewicz RZ, Rose JK A cell line expressing vesicular stomatitis virus glycoprotein fuses at low pH. *Science* 1984 Aug 17;225(4663):721-3; Riedel H, Kondor-Koch C, Garoff H Cell surface expression of fusogenic vesicular stomatitis virus G protein from cloned cDNA. *EMBO J* 1984 Jul;3(7):1477-83). Exposure of G protein to acidic pH induces a conformational change which allows the protein to interact simultaneously with the receptor and the target membrane, presumably via hydrophobic amino acids, to induce the membrane fusion (White JM. Membrane fusion. *Science* 1992 Nov 6;258(5084):917-24; White JM. Viral and cellular membrane fusion proteins. *Annu. Rev. Physiol.* 1990;52:675-97; Stegmann T, Doms RW, Helenius A Protein-mediated membrane fusion. *Annu. Rev. Biophys. Biophys. Chem.* 1989;18:187-211; Puri A, Winick J, Lowy RJ, Covell D, Eidelman O, Walter A, Blumenthal R Activation of vesicular stomatitis virus fusion with cells by pretreatment at low pH. *J Biol. Chem.* 1988 Apr

5;263(10):4749-53). It has been reported that incorporation of VSVG into liposome enhanced liposome-mediated gene transfection by 7-fold in vitro (Abe A, Chen ST, Miyanohara A, Friedmann T. In vitro cell-free conversion of noninfectious Moloney retrovirus particles to an infectious form by the addition of the vesicular stomatitis virus 5 surrogate envelope G protein. J Virol. 1998 Aug;72(8):6356-61). However, to our knowledge there are no reports that VSVG enhanced cationic polymer based gene carrier system gene transfer efficiency and reduced the cytotoxicity.

#### Summary of the Invention

10 In preferred embodiments, the present invention provides vectors and methods for transfecting eukaryotic cells, particularly higher eukaryotic cells, with nucleic acids. Nucleic acids, both DNA and RNA, linear or circular, are preferably introduced into cells such that they retain their biological function. The vector for transfecting eukaryotic cells preferably comprises a nucleic acid, a nucleic acid binding polymer, a lipid-based vesicle, 15 and a membrane active polypeptide, such as a viral envelope protein or a peptide derived from the envelope protein which retains functions of the viral envelope protein. Preferred vectors have significantly improved transfection efficiency and cytotoxicity as compared to similar vectors lacking a lipid-based vesicle and a membrane active polypeptide.

The nucleic acid may be deoxyribonucleic acid (DNA), ribonucleic acid (RNA) or a 20 DNA/RNA hybrid and may be in the form of a linear molecule or a circular molecule, such as a plasmid. The nucleic acid may be a single stranded oligodeoxynucleotide. An RNA may be a single or double-stranded RNA and may be a small interference RNA (siRNA) or a ribozyme.

Preferred transfection vectors contain nucleic acid binding polymers which self- 25 assemble in a complex with nucleic acids. These polymers effectively condense the nucleic acid and facilitate introduction of anionic macromolecules, like nucleic acids, through cell membranes, which are typically negatively charged. Preferred types of nucleic acid binding polymers include polymers that are linear, branched, star-shaped, grafted co-polymers, block copolymers, and dendrimers. A dendrimer may have more than three branches. The 30 nucleic acid binding polymer may have a molecular weight of 400 daltons (Da) or more. The polymers may be biodegradable or non-biodegradable. Preferred examples of biodegradable polymers include hydrolysable polymers, pH sensitive polymers, light

sensitive cleavable polymers, temperature sensitive cleavable polymers, sonication sensitive cleavable polymers, and enzymatically cleavable polymers. In some embodiments, the polymers are cationic polymers. Some examples of cationic polymers that may be used in preferred embodiments include poly-L-lysine (PLL), polyethelenimine (PEI), poly[a-(  
5 aminoutyl)-L-glycolic acid] (PAGA), chitosan, polyamidoamine (PAMAM), and poly(2-dimethylamino)ethyl methacrylate (pDMAEMA). Preferred complexes of nucleic acid and cationic polymer may contain various amounts of both components. Some embodiments of the invention have a ratio between cationic polymer and nucleic acid in the range of about 1:1 to 50:1.

Preferred transfection vectors comprise a vesicle having a native or synthetic lipid bilayer and a membrane active polypeptide that functions to facilitate entry of cationic polymer/nucleic acid complexes into a cell. In preferred embodiments, the native or synthetic phospholipid bilayer provides the microenvironment for fusion of the vector with the cell membrane. Various lipid mixtures may be used. One embodiment comprises a mixture of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). In a preferred embodiment, these three lipid components are in a ratio of about 6:2:2, respectively, by weight. Examples of membrane active polypeptides useful in transfection vectors are wild type viral envelope proteins and recombinant envelope proteins. Examples of viral protein include viral envelope vesicles, viral spike glycoproteins, multimers (e.g., dimers, trimers, or oligomers) thereof, peptides of viral spike glycoproteins, and envelope fragments containing embedded viral protein. In a preferred embodiment, vesicular stomatitis virus envelope protein, known as vesicular stomatitis virus glycoprotein (VSVG), is used. VSVG may be a multimer form, with multiple molecules of the protein together in a functional unit, or monomers of the protein, or a combination of both multimers and monomers; the VSVG may be wild type VSVG mature protein, a wild type VSVG peptide or a recombinant VSVG polypeptide. In some embodiments, transfection vectors comprising viral components of any enveloped viruses may be used. Other embodiments may have a non-viral protein as the membrane active polypeptide.

In preferred embodiments, the nucleic acid binding polymer and the nucleic acid form a complex. Such complexes may be formed spontaneously by adding the nucleic acid binding polymer to a nucleic acid solution at the ratio desired in the final product. After the

polymer has condensed the nucleic acid to form a complex, the complex is preferably combined with a lipid-based vesicle incorporating a membrane active polypeptide, preferably resulting in containment of the complex within the vesicle or binding of the complex by the vesicle. Accordingly, in one embodiment the complex of nucleic acid binding polymer and nucleic acid is encapsulated in a lipid-based vesicle with a membrane active polypeptide. In another embodiment, the complex of nucleic acid binding polymer and nucleic acid is bound to a lipid-based vesicle with a membrane active polypeptide.

Another embodiment provides a method for making a vector for transfecting a eukaryotic cell by isolating a nucleic acid, combining it with a nucleic acid binding polymer to form a complex, and combining the complex of nucleic acid and polymer with a solution containing lipid-based vesicles, where at least a portion of those vesicles has a membrane active polypeptide in contact with the vesicle. Some embodiments of this method use a cationic polymer as the nucleic acid binding polymer; in particular embodiments, a cationic polymer for use with this method may be poly-L-lysine, polyethylenimine, poly[a-(-aminoutyl)-L-glycolic acid], chitosan, polyamidoamine, or poly(2-dimethylamino)ethyl-methacrylate. Some embodiments use a biodegradable polymer in the making of a vector. Examples of preferred biodegradable polymers include hydrolysable polymers and pH-sensitive cleavable polymer. The hydrolysable polymer may be a biodegradable cationic polymer. The pH-sensitive cleavage polymer may be a polyacetal polymer. In preferred embodiments, lipid-based vesicles may contain native lipid membrane or synthetic lipid membrane. In some embodiments of the invention, the membrane active polypeptide is VSVG; in other embodiments, the membrane active polypeptide is a part of VSVG that retains activity with regard to membrane fusion.

Other embodiments include a method of gene therapy where an individual in need of gene therapy is identified and is administered a vector in a therapeutically effective amount. In preferred embodiments, the individual is a mammal.

Additional embodiments include a method of introducing a nucleic acid into a cell by contacting the cell with a vector as described herein. The cell is preferably a eukaryotic cell. Examples of eukaryotic cells that may be used include a human fibroblast, an animal embryonic stem cell, a keratinocyte, a pancreatic cell, a myocardium cell, a bone marrow cell, a neuronal cell, and a macrophage.

These and other embodiments are described in greater detail below.

### Brief Description of the Drawings

Figure 1 is a an illustration of the preparation of a functional vesicle, specifically a vesicular stomatitis virus glycoprotein (VSVG) vesicle.

5 Figure 2 is an image of a Western blot which resulted in the detection of VSVG from both cell lysate and cell conditioned medium preparations as described in Example 1.

Figure 3 shows the effects of VSVG vesicles on cationic polymer mediated gene delivery in HT1080 cells via  $\beta$ -galactosidase gene staining (for visualization) (3A and 3B) and in HepG2 cells (hepatic carcinoma cells) via luciferase activity measurement  
10 (quantification) (3C).

Figure 4 shows reproductions of FITC assays illustrating the effects of VSVG vesicles on cationic polymer mediated antisense oligonucleotide delivery in 293 cells. Figures 4A and 4B show antisense oligonucleotide delivery mediated by cationic polymer PEI or PLL alone. Figures 4C and 4D shows delivery by cationic polymer PEI or PLL with  
15 VSVG vesicle (250 ng). The antisense oligonucleotide was labeled with FITC fluorescent tag.

Figure 5 is a diagram illustrating a synthetic method for making biodegradable polymers.

### Detailed Description of the Preferred Embodiments

It has now been discovered that viral envelope components may be used to significantly enhance the efficiency of cationic polymer-mediated transfection of eukaryotic cells. In contrast to previous systems, the enhanced transfection systems described herein preferably include four major components: a nucleic acid binding polymer, a nucleic acid molecule, a native or synthetic lipid bilayer and a membrane active polypeptide. This invention is not bound by theory, but it is believed that the nucleic acid binding polymer interacts with nucleic acid molecules to spontaneously form a complex via ionic interactions. Condensing nucleic acid via the interaction with the nucleic acid binding polymer before introduction of the nucleic acid to a lipid component is believed to overcome the drawback of cationic lipid-based gene carrier systems that exhibit poor condensation efficiency. In preferred embodiments, condensed DNA/polymer complexes are encapsulated in native or synthetic lipid bilayer vesicles containing membrane active

polypeptides, also referred to herein as functional vesicles. It is believed that the native or synthetic phospholipid bilayer provides a microenvironment for vector fusion with a cell membrane. Membrane active polypeptides embedded in the lipid bilayer are believed to enhance the gene carrier system's ability to escape from endosomes, resulting in enhanced transfection efficiency. It is believed that the containment of the polymer/DNA complex within functional vesicles produces the reduced toxicity observed in preferred embodiments, as compared to other transfection systems. In addition, in those embodiments where functional vesicles are associated with VSVG or a membrane active fragment of VSVG, preferred methods of transfection may be applicable to a wider range of cell-types than previous techniques because the phospholipid receptor of VSVG is present on many cell surfaces. Preferably, specific ligand receptors in target cell lines are not required. In some embodiments, the VSVG present within the vector is in the form of a single polypeptide, which preferably enhances the stability of the vector. In preferred embodiments, the addition of VSVG vesicles to host cells enhances polymer-based gene transfection and reduces the cytotoxicity of nucleic acid binding polymers to the host cells in vitro, as compared to other systems.

Preferred embodiments provide improved methods for nucleic acid binding polymer-based gene transfer of nucleic acids and other biomolecules to eukaryotic cells. This invention is not bound by theory, but it is believed that this improvement results from the use of a functional vesicle having a native or synthetic lipid bilayer and a membrane active agent, to enhance the efficiency of transfection, to broaden the range of types of cells that can be transfected, to reduce cytotoxicity, or a combination of those effects. Preferred embodiments have significant advantages over methods used in previous transfection systems that employ viruses. With preferred embodiments, there is no limit on the size or composition of nucleic acid that may be transfected, no requirement for chemical modification of the nucleic acid, and no risk of virus contamination in the vector preparation, wild type virus regeneration, or tumorigenesis.

Preferred embodiments have significant advantages over viral-lipid systems. In preferred embodiments, nucleic acid binding polymer-based gene carriers may self-assemble without requiring additional reagents, in contrast to some previous transfection systems, such as a lipid gene carrier system. Preferred vectors are water soluble, stable and do not trigger an immune response. The risk of active virus contamination or wild-type

virus regeneration in other systems created by the use of whole virus as a reagent for enhancing transfection is avoided in preferred embodiments because no viruses or replication-competent viral genetic material are used in the preparation of the vectors.

Preferred embodiments comprise a lipid bilayer which is capable of maintaining the vector as an integrated whole. VSVG, for example, may be held in the bilayer and thus resistant to detachment from the vector by shearing forces which may be encountered when the vector is administered *in vivo*. Preferred embodiments of the vectors described herein have a lipid bilayer comprising a membrane active protein or peptide. In these embodiments, the bilayer maintains the vector as an integrated whole and prevents disassociation of the membrane active portion from the vector.

Preferred embodiments also have advantages over previous transfection methods mediated by lipid-based gene delivery systems. The pretreatment of nucleic acid with cationic polymer before the introduction of the lipid vesicle in preferred embodiments overcomes the drawback of poor nucleic acid condensation by the lipid used in previous transfection methods, resulting in enhanced transfection efficiencies.

Preferred embodiments also have advantages over previous transfection methods wherein the gene carrier systems employ cationic polymer alone. In preferred embodiments, inclusion of a functional vesicle with membrane active polypeptide, protein or derived peptides in a transfection vector comprising nucleic acid binding polymer in a complex with nucleic acid significantly enhances transfection compared to the transfection mediated by the nucleic acid binding polymer with nucleic acid alone. For example, addition of VSVG envelope vesicles to a complex of PLL/DNA was found to enhance gene transfer by up to 1000 times. Incorporation of VSVG vesicle to PEI/pDNA complexes was found to enhance gene transfer efficiency in HT1080 human fibrosarcoma cells by up to 8 to 10 times. Inclusion of VSVG vesicles was found to increase the rates of gene transfer by commercially available dendrimer polymer-mediated gene transfer systems by 7 times. Enhancement of transfection by VSVG vesicles was also demonstrated in primary cell lines that have been found to be difficult to transfect by known cationic polymer-mediated transfection methods. Enhancement of transfection by functional vesicles with virally derived membrane active polypeptides may occur in any cells that the virus can enter and infect in its normal life cycle, without risk of virus contamination. Enhancement of

transfection by vesicular stomatitis virus may occur with a wide range of cells, particularly cells which contain "rich receptors", such as acidic phospholipid phosphatidylserine

Preferred embodiments do not require the use of a ligand that binds to receptors on the surface of target cells and nucleic acid-complexing agents need not be chemically linked to such ligands nor to functional vesicles. Such embodiments are useful for transfection of a wider range of cell types, including types that are difficult to transfect with previous transfection methods. Preferred embodiments employ, for instance, envelope vesicles of rhabdovirus, preferably vesicular stomatitis virus envelope vesicles, that are functional with a wide range of higher eukaryotic cells, both from vertebrates and invertebrates, including 5 mammalian, avian, reptilian, amphibian and insect cells. Preferred embodiments that employ, for example, envelope vesicles of rhabdovirus, preferably vesicular stomatitis virus envelope vesicles, significantly reduce the cytotoxicity associated with cationic polymer gene carrier-mediated gene transfection. This reduction in cytotoxicity is believed to be due to receptor-mediated endocytosis of viral envelope, which assists in the entry of the 10 vector contents into the cell, and the protection of the cationic polymers by the lipid 15 bylayers of the vector.

As used herein, the term "nucleic acid binding polymer" means an organic polymer that interacts with a nucleic acid to form a physical association with the nucleic acid. Such interaction may be, for example, an attraction due to charge differences between the 20 polymer and the nucleic acid. Nucleic acid binding polymers are preferably cationic polymers. Preferred cationic polymers include linear and branched polymers, biodegradable and non-biodegradable polymers, polymers with or without conjugation with other functional groups, and with or without lipid incorporation or conjugation. Preferred 25 embodiments include cationic polymers that are capable of forming complexes with nucleic acid.

The term "transfection" is used herein generally to mean the delivery and introduction of biologically functional nucleic acid into a cell, e.g., a eukaryotic cell, in such a way that the nucleic acid retains its function within the cell. Transfection encompasses delivery and introduction of expressible nucleic acid into a cell such that the 30 cell is rendered capable of expressing that nucleic acid. The term "expression" means any manifestation of the functional presence of the nucleic acid within a cell, including both transient expression and stable expression. The term "nucleic acid" encompasses both

DNA and RNA without size limits from any source comprising natural and non-natural bases. Nucleic acids may have a variety of biological functions. They may encode proteins, comprise regulatory regions, function as inhibitors of gene or RNA expression (e.g., antisense DNA or RNA or RNAi), function as inhibitors of proteins, function to inhibit cell growth or kill cells, catalyze reactions, or function in a diagnostic or other analytical assay.

5 Nucleic acids used in preferred embodiments may be in a variety of forms. They may be single stranded, double stranded, branched or modified by the ligation of non-nucleic acid molecules. They may be in a linear form or a closed circle form. In some embodiments, plasmid DNA is used as the nucleic acid. Plasmid DNA is a variety of closed circular DNA

10 and preferably contains a bacterial origin of replication or an equivalent sequence that allows the replication of the DNA molecule in a biological system.

The term "lipid-based vesicle" means a small (subcellular) container having walls made up of lipids. The lipids may be arranged in monolayers or bilayers. Preferred embodiments include lipid based vesicles where a lipid bilayer covers, contacts or 15 encapsulates a complex of polymer and nucleic acid. In some embodiments, lipid based vesicles contain other molecules in addition to the lipid molecules. For example, lipid based vesicles may contain membrane active polypeptides in some embodiments. In particular embodiments, the membrane active polypeptides associated with lipid-based vesicles are viral envelope proteins, such as VSVG. A membrane active polypeptide or 20 agent is a polypeptide or other biomolecule in a lipid-based vesicle that interacts with a cellular membrane to increase the likelihood of fusion of the lipid-based vesicle with the cellular membrane being contacted. The lipid-based vesicle containing such a membrane active polypeptide or agent may be referred to herein as a functional vesicle.

The term "cytotoxicity" refers to the loss of cell viability after cell exposure to a 25 component or a solution of a gene delivery system. In preferred embodiments, transfection methods employ cationic polymers in combination with vesicular stomatitis virus envelope vesicles composed of VSVG protein and lipid bilayer cell membrane. The methods of these embodiments have been shown to significantly enhance transfection (e.g., 1000 fold compared to PLL alone) over previous transfection methods that employ comparable 30 cationic polymers without a lipid vesicle.

Functional vesicles are believed to facilitate the entry into a cell of a gene carrier, such as a cationic polymer/DNA complex, and/or the release of gene carriers from

compartments or organelles within the transfected cells. In some embodiments, a membrane active polypeptide serves to promote the transport of complexes of polymer and nucleic acid into endosomal or other compartments within the cell being transfected. In some embodiments, membrane active agents include proteins, peptides and other molecules which may facilitate fusion of a gene carrier to a cell membrane and/or the penetration of a cell membrane to facilitate transport of the gene carrier into the cell. Preferably the peptides or biomolecules on the surface of the vesicles are derived from viral envelope protein, but may also include non-viral proteins or peptides that have equivalent or similar functions as viral envelope proteins.

Transfection activity or efficiency may be measured by detecting the presence of the transfected nucleic acid in a cell. Preferably the presence of a transfected nucleic acid is detected by measuring the biological function of the peptide encoded by the nucleic acid in the cell. More preferably, it is assessed by measuring the level of transient or stable expression of a reporter gene contained in the transfected nucleic acid. The level of reporter gene expression may depend on, among other things, the amount of nucleic acid transfected and on the level of activity of the reporter gene's promoter in the type of cell being transfected. Generally, there are two classes of reporter gene detection systems used for reporter gene assays to determine gene transfer efficiency: quantitation and visualization. Quantitative methods use the appropriate substrates to measure a reporter gene product's activity. For example, the bioluminescent enzyme luciferase catalyzes the oxidative carboxylation of beet luciferin, emitting photons that may be measured using a luminometer. The amount of luciferase activity is usually proportional to the overall efficiency of transfection for a cell sample. In one common approach to measuring luciferase activity in a sample of transfected cells, cell extracts are prepared and the amount of luciferase activity in the extract is determined. Measurements of the activities of reporter gene products may be used in turn to determine the gene transfection efficiency. Transfection activity may also be assessed by determining the percent of cells in a sample that have been transfected. With these techniques, individual cells are visualized under a microscope and the number of cells exhibiting characteristics of the transfected reporter gene are counted. For example, cells transfected with the reporter gene  $\beta$ -galactosidase undergo X-gal staining, during which the  $\beta$ -galactosidase present in a cell will hydrolyze X-

gal (5-bromo-4chloro-3-indoyl- $\beta$ -D-galactopyranoside) and yield a blue precipitate. Other detection and quantitative methods which may be used are well known in the art.

Preferred embodiments include methods useful for the transfection of cells that have commonly been difficult to transfet by previous techniques. These previous techniques 5 include those that use cationic polymers, such as poly-L-lysine. The term "difficult to transfet" refers to those eukaryotic cell lines in which, under transfection assay conditions as described in Example 3, less than about 1% of the cells in a sample are transfected employing the cationic polymer reagent poly-L-lysine alone. "Difficult to transfet" cells include animal primary cell lines such as human fibroblasts, animal embryo stem line cells, 10 keratinocytes and macrophages.

Preferred embodiments provide methods comprising contacting a eukaryotic cell with a transfection vector comprising a cationic polymer, a functional vesicle containing native or synthetic lipid bilayer, and a nucleic acid or other biomolecule. The functional vesicle may comprise an envelope of a vesicular stomatitis virus, an alphavirus, or an 15 influenza virus or a component thereof. Enhanced transfection methods of these embodiments have been demonstrated with the prototype envelope vesicle from vesicular stomatitis virus envelope vesicle (VSV-G vesicle) and the prototype vesicular stomatitis virus G protein (VSVG). VSVG has three domains: cytoplasmic, transmembrane, and extracellular. The extracellular domain is the fusogenic portion of the protein and has 20 the functions of recognizing a receptor or target on the surface of a cell, fusing to the cell and/or penetration of the cell membrane.

In preferred embodiments, a cationic polymer forms a cationic polymer/nucleic acid complex. Preferably, the cationic polymer spontaneously form a complex with nucleic acid in aqueous solution. Various well-known techniques may be employed to produce a 25 desired type of cationic polymer/nucleic acid complex. The relative amounts of cationic polymer employed to form the complexes with nucleic acid depends on the type of complex desired (surface charge, complex size and shape), the toxicity of the cationic polymer to the cell, and the environment (e.g., medium) in which the polymer is to be employed. The kinds and amounts of cationic polymer employed are typically balanced to minimize cell 30 toxicity and maximize transfection efficiency. In preferred embodiments, the cationic polymer forms a complex with the nucleic acid that is to be transfected into cells. Preferably, nucleic acid complexes are formed by combining the nucleic acid with the

cationic polymer prior to functional vesicle addition. Nucleic acid/cationic polymer complexes may then be encapsulated within functional vesicles via physical and chemical methods.

In preferred embodiments, transfection vectors include functional vesicles composed of a native or synthetic lipid bilayer and a biomolecule including viral envelope protein, or components thereof. The viral envelope protein may be wild-type, mutant, or genetically modified. In preferred embodiments, mutant or genetically modified envelope proteins retain the ability to enter eukaryotic cells. Some previous gene transfection techniques use whole virus in some form, including wild type virus, replication-deficient virus or virus inactivated by a variety of methods. Preferred embodiments avoid the safety risks and immune complications typical of these previous techniques by not utilizing whole virus in any form. In preferred embodiments, the production of viral envelope proteins requires only an envelope gene that has been isolated in some way. In some embodiments, the viral envelope gene is cloned into a mammalian gene expression plasmid. This plasmid, once transfected into cultured cells, will produce the viral protein. In preferred embodiments, in contrast to some previous techniques, there is no risk of regeneration of wild-type virus or of immunogenicity complications in a individual because the intact viral genome is not involved in the production of the envelope protein or the envelope vesicles, thus there are no viral proteins included in the envelope vesicles, other than molecules of the envelope protein.

Preferred embodiments use viral envelope components, especially viral envelope vesicles and viral envelope proteins, to enhance gene transfection efficiency and reduce cytotoxicity. The functional vesicles may include a native cell membrane or synthetic lipid bilayer, and may include membrane active polypeptides. Some examples of membrane active polypeptides include spike glycoproteins, multimers of spike glycoproteins (dimers, trimers or oligomers) and peptides of spike glycoproteins, any of which may function to enhance non-viral gene transfection into cells. Any proteins or peptides that have functions similar to viral envelope proteins described herein may be incorporated into lipid bilayer vesicles to encapsulate cationic polymer-nucleic acid complexes to achieve enhanced transfection and reduced cytotoxicity in particular embodiments.

In preferred embodiments, viral envelope components may be isolated by a variety of well-known techniques. The use of gradient ultracentrifugation for isolation of cellular

components, as described in Abe, A. et al., In Vitro Cell-Free Conversion of Noninfectious Moloney Retrovirus Particles to an Infectious Form by the Addition of the Vesicular Stomatitis Virus Surrogate Envelope G Protein. *J. Viology* 1998, 72:6356-6361; and the use of spin filters and the cationic detergent DTAB for the isolation and purification of viral protein fractions, as described by Glushakova, et al., Isolation of influenza virus hemagglutinin and its separation into subunits by a stage-by-stage scheme for viral protein fractionation, *Vopr Virusol.* 1988, 33(3):286-9, are two known methods that can be adapted for the isolation of VSVG vesicles. Alternatively, membrane active polypeptides may be produced by a variety of standard genetic engineering synthesis methods. The peptide or biomolecule may be purified by an affinity column with the addition of tag or another ligand. In one variation of this method, the viral envelope gene within an expression plasmid is immediately preceded or followed by a genetic sequence that codes for a small peptide tag. The affinity column contains an antibody to the peptide tag and thus the column will bind the hybrid protein. The hybrid protein is eluted from the column, the tag is removed by enzymatic action and the viral envelope peptide is recovered. The viral functional peptides derived from envelope proteins also may be produced by standard chemical synthesis using automated solid phase peptide synthesis. It is apparent to those with ordinary skill in the art that a variety of methods may be used to generate membrane active polypeptides.

Media employed in transfection experiments done in accordance to some embodiments is similar to the medium used to culture cells for transfection. In preferred embodiments, media containing serum will have no significant effect on the efficiency of transfection. This simplifies the procedure of transfection for these embodiments and reduces the risk of contamination due to the extra steps of changing the transfection medium.

A variety of cationic polymers are known in the art. Examples of cationic polymers useful in this invention are listed in Table 1. Useful cationic polymers include those with a molecular weight over 400 Da, either linear or branched, biodegradable or non-biodegradable, with modification or without modification and with lipid conjugation or without lipid conjugation. Particular embodiments use polymers that are block copolymers or grafted copolymers.

It has been found that the following parameters may affect performance in a particular case: cationic polymer concentration, the molecular weight of cationic polymer, the concentration of nucleic acid, the methods of forming functional vesicles, the medium employed for transfection, the length of time the cells are incubated with transfection composition, the amount of functional vesicle or viral component employed, the ratio of each component in the complexes and the way in which the components of the transfection composition are combined into cationic polymer/DNA complexes. Routine experimentation, using the guidance provided herein, may be carried out to identify the proper parameter for a particular cell to be transfected.

It will also be apparent to those of ordinary skill in the art that methods, reagents, procedures and techniques other than those specifically detailed herein may be employed or readily adapted to produce the transfection vectors of the present invention and practice the transfection methods of this invention. Such alternative methods, reagents, procedures and techniques are within the spirit and scope of this invention.

The transfection compositions and methods of this invention are further illustrated in the following non-limiting Examples. All abbreviations used herein are standard abbreviations in the art. Specific procedures not described in detail in the Examples are well-known in the art.

## EXAMPLES

### Cell Cultures and Plasmids

Standard tissue culture methods were employed. Human embryonic kidney transformed HEK 293T cells were maintained in Dulbecco's Modified Eagle Media (DMEM) (Gibco Inc.) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. In this media the cells had a doubling time of about 20 hours, and the cells were split every 3-4 days to avoid confluency.

The HeLa 705 cell line was derived from Human cervical carcinoma HeLa cells by introducing a firefly luciferase gene with a mutant β-globin intron (a mutation at 705 position) that expresses an inactive protein due to the incorrect splicing. However, the mutated intron can be corrected by a specific antisense oligonucleotide which blocks the mutant splicing site (Kang SH et al. Biochemistry 1998;37(18):6235-9). The cell line was maintained in DMEM (Gibco) containing 10% fetal bovine serum, 100 units/ml penicillin

and 100 µg/ml streptomycin. Two hundred µg /ml hygromycin was added into medium to maintain the luc-705 plasmid. In this media the cells had a doubling time of about 20 hours and were split every 3-4 days to avoid confluence.

Human liver tumor cell line HepG2 was maintained in α-MEM medium (Gibco, 5 Inc.) containing 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. In this media the cells had a doubling time of about 20 hours, and the cells were split every 3-4 days to avoid over confluence.

Human primary endothelial cell HUV-EC cell line was grew and maintained in EBM medium (Cambrex Corp.) containing 10% fetal bovine serum, 100 units/ml 10 penicillin, 100 µg/ml streptomycin, and various growth factors as specified by the manufacturer.

Bovine artery endothelial cells (BAEC) and bovine aorta smooth muscle cells (BASMC) were isolated from bovine aorta and prepared as described according to known methods (Yu, L., Nielsen, M., and Kim, SW. TerpelxDNA Gene Carrier System Targeting 15 to Artery Wall Cells. J. Controlled Release 72:179-189 (2001)). Briefly, aortas were taken from bovine cadavers at a slaughterhouse (Dale Smith and Sons, Draper, UT). The endothelial cell (EC) cultures were prepared by luminal digestion with 0.3% collagenase in PBS. The smooth muscle cell (SMC) cultures were prepared by dissection and enzymatic digestion with 0.3% collagenase and 0.4% elastase in DMEM. EC cells are supplemented 20 with basic fibroblast growth factor (bFGF) for optimal growth and expression of normal cobblestone morphology. Cells used in transfection experiments were fed culture media (DMEM with 10% FBS) containing 20 ng bFGF/ml for at least five days prior to their use in a transfection assay. These cells were then trypsinized and plated out for transfection experiments. For a transfection experiment these cells were plated so that they formed an 25 incomplete monolayer (70-80 % confluence). SMC were cultured with DMEM medium containing 10% FBS. These cells were passaged in the same way as the ECs.

The plasmid pMNK-VSVG was constructed by cloning VSVG cDNA into plasmid pMNK with standard molecular methods. The expression of VSVG cDNA is controlled by 30 human cytomegalovirus (CMV) promoter and the transcripts are stabilized by a gene expression enhancer, chicken β-globulin intron. The plasmid vectors pCMV-lacZ, pCMV-GFP and pCMV-luc were constructed by cloning the *E. coli* β-galactosidase gene, green fluorescent gene and firefly luciferase gene into pCMV-0, with the same backbone of

mammalian expression vector, pMNK-VSVG, respectively. Plasmid DNA was amplified and purified with Qiagen EndoFree Plasmid Max Preparation Kid according to the manufacturer's instruction.

5    Biodegradable polymer synthesis

Synthesis of branched or slightly cross-linked biodegradable cationic polymers is illustrated in Figure 5. This synthesis method can be used for preparation of large libraries of branched or slightly crosslinked biodegradable cationic polymers.

For example, in a preferred embodiment, A may represent an amine-containing 10 cationic compound or oligomer with at least three reactive sites (for Michael addition reaction), and B may represent a compound having at least two acrylate groups (see Figure 5). The polymerization reaction between A and B takes place under very mild conditions in organic solvents. After the reaction, the polymers can be recovered by at least two different methods. In the first method, the polymers may be recovered by direct removal of the 15 solvents at reduced pressure. In the second method, the polymers may be neutralized by adding acid, such as hydrochloric acid, and the neutralized polymers recovered by filtration or centrifugation. Branched or slightly cross-linked, water soluble polymers with high molecular weight can be obtained by controlling the ratio of A to B, reaction time, reaction temperature, solvents, and concentration of the solutes.

20

Polymers prepared by crosslinking cationic oligomers with diacrylate linkers, recovered by direct removing solvents

Synthesis of high molecular weight cationic polymers may be performed by a variety of methods known to those of ordinary skill in the art. The synthesis of a polymer 25 which is derived from polyethylenimine oligomer with molecular weight of 600 (PEI-600) and 1,3-butanediol diacrylate (1,3-BDODA) is provided as a general procedure to serve as a model for other synthetic procedures involving similar compounds which can be used to synthesize other cationic polymers. 0.44g of PEI-600 (Aldrich) was weighed and placed in a small vial, and 6 ml of methylene chloride was added. After the PEI-600 completely 30 dissolved, 0.1g of 1,3-BDODA in 2 ml of methylene chloride was added slowly into the PEI solution while stirring. The reaction mixture was stirred for 10 hours at room temperature. After removing the organic solvent under reduced pressure, 0.55g of

transparent, viscous liquid was obtained.  $^1\text{H-NMR}$  spectrum indicated that the acrylic carbon-carbon double bond disappeared completely. The molecular weight of the obtained polymer was estimated by agarose gel electrophoresis. Several biodegradable cationic polymers (BCP-1, BCP-2, and BCP-3) were prepared in a similar manner and used in the 5 transfection procedures described below, the results of which are shown in Tables 2-5, 7, and 8. Other branched or slightly crosslinked, degradable cationic polymers derived from other cationic oligomers and other linkers having structures similar to those used herein were prepared in a similar manner.

10

### EXAMPLE 1

#### VSVG vesicle preparation by biological method

The plasmid pMNK-VSVG was transfected into 293 cells by superFect<sup>TM</sup> (Qiagen, Valencia CA) according to the manufacturer's instructions. Two days after transfection, VSVG vesicles were prepared by two methods. One method involved harvesting 15 conditioned medium from 293 cells that were transfected with plasmid containing VSVG gene, pMNK-VSVG, filtering through (0.45  $\mu$ ) filter, followed by centrifugation at 30,000 rpm with a SW35 rotor for 60 min at 4°C. The pelleted VSVG vesicles were resuspended in phosphate-buffered saline (PBS) (pH 7.4), and the same volume of 60% sucrose PBS solution was added, followed by layering 4 ml of 20% and 10% of sucrose PBS solution 20 and centrifuged at 30,000 rpm for 30 min at 4°C. The fractions containing VSVG vesicles were collected and dialysed against PBS for three changes for 20 hrs at 4°C.

The second method involved physically breaking the cell membrane, followed by sucrose gradient ultracentrifugation separation. Briefly, pMNK-VSVG plasmids transfected 293 cells were treated with latex beads (polybead polystyrene microspheres 4.55 25 x 10 beads/ml, polysciences Inc.) for 1-2 hours prior to harvesting. After the bead treatment, the media was aspirated and the cells washed one time with 10 to 20 ml of buffer 1 ( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  free Phosphate Buffered Saline (PBS) solution, buffered with 0.02M Hepes pH=7.4). Cells were harvested by putting 20 ml of buffer 2 ( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  30 free PBS, buffered with 0.02 M Hepes, and 1mM EDTA, pH=7.4) on each plate for 10 min in a 37°C incubator. The cells were removed from plates by pipeting up and down, were transferred to a 50 ml tube and centrifuged at 4°C at 150 x g for 10 min.

All following steps were done at 4°C. The cell pellets were resuspended in 25 ml of PBS and the sample was transferred to tubes with 20 ml of 4% BSA PBS. The samples were centrifuged at 4°C at 300 g for 15 min. The cell pellet was washed two times with PBS, followed by centrifugation at 4°C at 150 g for 5 min for each wash. The cells were 5 homogenized by a Dounce Homogenizer vigorously for 15 min. Five milliliters of 60% sucrose was added to the 5 ml of homogenate.

A volume of 3.330 ml of the homogenate-30% sucrose mixture was put into Beckman ultra clear centrifuge tubes and layered with 6 ml of 20% sucrose solution and 3 ml of 10% sucrose solution on the top. The samples were centrifuged in SW41 rotor 10 buckets at 4°C at 110,000 g (30,000 RPM) for 90 min. VSVG membrane fractions, found between the 10% and 20% sucrose layers, were collected and subjected to a second centrifugation in SW41 rotor buckets at 4°C at 8000 RPM for 30 min. The VSVG vesicles were resuspended in 500  $\mu$ l of 10% sucrose and stored at -70°C. The procedure for VSVG preparation is summarized in Figure 1. Figure 1 shows the general procedures of making 15 VSVG vesicles from 293 cells (Human Embryonic Kidney Cell). The plasmid DNA carrying VSVG gene was transfected into 293 cells and after 24 to 48 hours culture, the cells and medium were harvested separately through centrifugation. The pellets of VSVG vesicle were further separated from other components by gradient centrifugation methods. The prepared VSVG vesicle can be directly used in *in vitro* cationic polymer based 20 transfection assays. VSVG concentration was determined by Coomassie plus protein assay kit (PIERCE, Rockford, IL) and VSVG was identified by immunoblotting analysis, as seen in Figure 2, according to standard molecular biology protocol. Figure 2 shows the VSVG proteins purified from cell lysate or cell conditioned medium (supernatant) being identified by Western blot assay with an anti-VSVG antibody probe.

25

#### EXAMPLE 2

##### VSVG vesicle preparation by synthetic method

Chemicals: All Fmoc amino acids and rink amide MBHA resins were purchased from Nova Biochem. Dimethylformamide (DMF), piperidine, dichloromethane (DCM), 1-hydroxybenzotriazole (HOBt), 1,3-diisopropylcarbodiimide (DIC), N,N-diisopropylethylamine (DIPEA), diethyl ether, trifluoroacetic acid (TFA), triisopropylsilane (TIS), and acetic anhydride were obtained from Aldrich. Egg PC, brain PE, brain PS, and

Triton X-100 were purchased from Sigma. PEI 1800 and PEI 25000 were supplied by Polysciences.

Peptide synthesis and conjugation: Peptides were synthesized by the standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) solid-phase method on the rink amide MBHA (4-Methylbenzhydrylamine HCl) resins. Briefly, the resins were swelling in DMF for 30 minutes. The Fmoc group was removed by treating the resins with 20% piperidine in DMF for 10 minutes. The resins were washed with DMF, DCM, and DMF, respectively. Then, amino acid previously dissolved in the mixture of DMF with HOBr was added to the resins. DIC and DIPEA were also added to the solution in order to form the OBr ester bond. Normally, the reaction was completed within 2 hours. Acetic anhydride and DIPEA were added to block any possibly uncompleted portions on the resin beads. The resins were subsequently washed with DMF, DCM, and diethyl ether. The whole process was repeated from the removal of Fmoc group until all amino acids were added. The peptide-resin conjugates were then cleaved with a mixture of TFA/water/TIS (95:2.5:2.5). The crude peptides were dried under vacuum overnight.

Solid-phase peptide conjugation was conducted by following the dimethyl sulfoxide-mediated oxidation method. Briefly, the peptides attached to the resins were treated with 1% TFA in DCM containing 5% TIS. The resins were washed with DMF, DCM, and diethyl ether. Additional crude peptides to be added to the peptide-resin were dissolved in 5% acetic acid in water and then added to the peptide-resin beads. The pH of solution was adjusted to 6 with ammonium carbonate and dimethyl sulfoxide was then added. The reaction was completed after 24 hours. The conjugated peptides were cleaved from the resins with a mixture of TFA/water/TIS (95:2.5:2.5). The conjugated peptides were then dried under vacuum overnight.

Vesicle formation: Vesicles were prepared from mixtures of egg PC/brain PE/brain PS at a ratio 6:2:2 (w/w/w). Peptides (peptide:lipid = 1:100 mol/mol) previously dissolved in trifluoroethanol and cationic polymers (PEI 1800 or PEI 25000) previously dissolved in PBS were added to the egg PC/brain PE/brain PS mixture. GFP reporter gene was added to resulting mixture, followed by Triton X-100 (0.005% w/w). The solvent was evaporated under argon gas until a lipid film was obtained at the bottom of the flask. The film was then further dried under vacuum overnight. The lipid film was rehydrated in the buffer containing 10 mM Tris and 250 mM NaCl, pH 7.5, by shaking at 37°C for 1 hour. The

vesicle suspension was sonicated in a bath sonicator; the lipid suspension began to clarify and yielded a slightly hazy transparent solution within 40-60 minutes. During the sonication, the temperature in the water bath was maintained under 30°C to prevent deterioration of liposomes. The lipid aggregates were then removed by centrifugation at 5 16,000 rpm for 20 minutes to yield a clear solution of small, unilamellar vesicles (SUVs). The resulting SUVs were used for gene transfection study in cell cultures.

Preparation of peptide vesicle-GFP reporter gene complexes: The peptide vesicle-GFP reporter gene complexes were freshly prepared before performing the experiment. Briefly, the plasmid containing GFP gene or antisense oligonucleotide with fluorescent dye 10 tag (FITC) was added directly to the peptide SUVs. The mixture was incubated at room temperature for 10 minutes before use. Various amounts of Triton X-100 were added to the mixture to aid solubility. The peptide-lipid vesicle was constituted after dialysis against PBS pH 7.2.

Transfection of peptide vesicle-GFP reporter gene complexes in cell cultures: Cos-15 7 cells were seeded in 96-well plate the night before conducting the study to obtain the cell density about 60-70%. The medium was discarded and the cells were washed with PBS once before adding the flash prepared lipid-peptide vesicles with reporter plasmid, pCMV-GFP. After being incubated in 37°C CO<sub>2</sub> incubator for 6 hours, the medium containing the vesicles were replaced by the Dulbecco's Modified Eagle Medium with 10% FBS. The 20 cells were kept in 37°C CO<sub>2</sub> incubator for 24 hours before observing the signal under the microscope.

### EXAMPLE 3

#### Transfection Assays in 293 and HT1080 Cells By Adding VSVG Vesicles

25 PLL, PEI, polyamidoamine (PAMAM, dendrimer) and biodegradable polymers were used for transfection of plasmid DNA into mammalian cells in vitro to evaluate the effect of VSVG vesicles on cationic polymer mediated gene transfer, as is illustrated in Figure 3. The cultured cells (from cell lines 293 and HT1080) were plated in 24-well tissue culture plates (1X10<sup>5</sup> cells/well for 293 cells and 5X10<sup>4</sup> cells/well for HT1080) and 30 incubated overnight in DMEM with 10% FBS. The primary cells (bovine aorta endothelial cells (6X10<sup>4</sup> cells/well) and bovine aorta smooth muscle cells and 3X10<sup>4</sup> cells/well) were plated in 24-well tissue culture plates and incubated overnight in DMEM with 10% FBS and 20 ng bFGF/ml. For each well, an aliquot of 100-11 DNA solution containing 1 µg of

plasmid DNA, e.g. pCMV-lacZ plasmid DNA or pCMV-luc, was mixed with 100  $\mu$ l cationic polymer solution containing 2  $\mu$ g of PLL or 0.25  $\mu$ g of PEI. The DNA and cationic polymer solutions were mixed and incubated for 10-15 min at room temperature to allow the formation of DNA-cationic polymer complexes. Various amounts of VSVG vesicles in 5 100  $\mu$ l of 10% glucose solution were added to the DNA-cationic polymer complex-containing solutions and were then added to the cells in individual wells after the cells were washed with PBS. Cells were incubated (37° C, 5% CO<sub>2</sub>) for 24 hrs without changing the medium, after which they were assayed for *E. coli* beta-galactosidase and fruitfly luciferase activities using the methods described below.

10  $\beta$ -galactosidase activity Cytochemical assay

In situ staining was used to demonstrate *E.coli* beta-galactosidase gene expression as a standard procedure. Cells were rinsed with PBS, fixed for 5 min in 2% (v/v) formaldehyde, 0.2% glutaraldehyde in PBS, rinsed twice with PBS, and stained 2 hours to overnight with 0.1% X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 15 mM MgCl<sub>2</sub> in PBS. Rinsed cells were photographed using a 10X objective on a Olympus inverted microscope. The percent of stained cells in transfected cultures was determined from counts of three fields for optimal cationic polymer amounts. The results of transfections of 293, HT1080 and bovine artery wall primary cells with pCMV-lacZ using various transfection reagents are presented in Figure 3. Figure 3 shows that the effects of 20 VSVG vesicles on cationic polymer-mediated gene delivery in HepG2 cells (hepatic carcinoma cells) via staining for  $\beta$ -galactosidase with X-gal (for visualization) and via luciferase activity measurement (quantification). With the VSVG vesicles, the number of  $\beta$ -galactosidase-positive cells transfected by cationic polymer (poly-L-lysine, PLL) (Fig. 3B) increased to 20 to 25%, compared to the less than 5% positive cells for transfection 25 with PLL alone (Fig. 3A). Using a luciferase reporter gene assay, the VSVG vesicles were shown to enhance PLL-mediated gene transfection efficiency from 10<sup>4</sup> to 10<sup>7</sup>, three orders higher, as compared to PPL-mediated transfection without VSVG vesicles (Fig. 3C). Ratios given in Fig. 3C are vesicle:PLL:plasmid DNA.

GFP Reporter Gene Transfection assay

30 Green fluorescent protein (GFP) gene was used in an initial screening. After transfection, the GFP signal in cells was observed under fluorescent microscope (Olympus, filter 520nm). Cells were photographed using a 10X objective. The percentage of cells

with GFP signal in transfected cultures was determined from counts of three fields for optimal cationic polymer amounts. The results of experiments done to study the effects of VSVG vesicles on the transfection of GFP constructs are found in Tables 3 and 4 below.

Luciferase Activity Assay

5 Measurement of luciferase activity was performed using a chemiluminescent assay following the manufacturer's instructions (Luciferase Assay System; Promega, Madison, WI, USA). Briefly, thirty hours after gene transfer, the cells were rinsed twice with PBS and then were lysed with lysis buffer (1% Triton X-100, 100 mM K<sub>3</sub>PO<sub>4</sub>, 2 mM dithiothreitol, 10% glycerol, and 2 mM EDTA pH 7.8) for 15 min at room temperature. A  
10 20- $\mu$ l aliquot of cell lysate was then mixed with 50- $\mu$ l of luciferase assay reagent with injector at room temperature in the luminometer. Light emission was measured in triplicate over 10 seconds and expressed as RLU (relative light units). Relative light units (RLU) were normalized to the protein content of each sample, determined by BCA protein assay (Pierce, Rockford, Illinois). All the experiments were conducted in triplicate. The results of  
15 transfection of 293, HT1080 and bovine artery wall primary cells with pCMV-luc using various transfection reagents are presented in Tables 1 and 2.

Table 1. The effect of VSVG Vesicles on Cationic Polymer Mediated Gene Transfer

Transfection Efficiency (RLU/mg of protein)		
In Vitro in 293 cells		
Polymers	Without VSVG	With VSVG
PLL	25000+-12000	13000000+-6500000
PEI	670000+-140000	45000000+-3400000
Dendrimer	950000+-230000	56000000+-7600000
HT1080 cells		
PLL	15000+-16000	11000000+-3500000
PEI	330000+-90000	23000000+-800000
Dendrimer	760000+-270000	41000000+-3800000
Bovine Endothelial Cells		
PLL	160+-80	32000+-13000
PEI	9200+-7800	66000+-35000
Dendrimer	11000+-8700	72000+-54000
Bovine Smooth Muscle Cells		
PLL	120+-98	31000+-24000
PEI	8500/7600	112000+-65000
Dendrimer	21300+-15000	106000+-35000

The data in Table 1 show that the effect of VSVG vesicles on enhancing cationic polymer mediated gene transfer in various cell lines including transformed cells (293 cells and HT1080 cells) and primary cells (bovine endothelial cells and bovine smooth muscle cells). The transfection efficiencies were measured by luciferase activities. Three cationic polymers were selected in these studies, including linear cationic polymer with only primary amine groups (PLL), the branched cationic polymer with primary, secondary, and tertiary amine groups (PEI), and cationic dendrimer. With VSVG vesicle help, the cationic polymer mediated transfection efficiencies showed at least 100 times higher compared to that with cationic polymer alone.

Table 2. The Effect of VSVG Vesicles On Degradable Polymer Mediated Gene Delivery

Transfection Efficiency (RLU/mg of protein)		
In Vitro in 293 cells		
Polymers	Without VSVG	With VSVG
BCP-1	2,500,000 +/- 120,000	27,000,000 +/- 6,500,000
BCP-2	6,700,000 +/- 140,000	72,000,000 +/- 340,000
BCP-3	9,500,000 +/- 230,000	110,000,000 +/- 7,600,000

The data in Table 2 show that VSVG vesicles also enhanced biodegradable cationic polymer mediated transfection efficiencies significantly (at least 10 times higher) in 293 cells with luciferase reporter gene.

Table 3: The Effect of Synthetic VSVG Vesicles On Cationic Polymer Mediated Reporter Gene GFP Delivery

Transfection Efficiency (GFP Positive Cells%)		
In Vitro in COS7 cells		
Polymers	Without Synthetic Vesicle	With Synthetic Vesicle
PLL <sub>25k</sub>	3% +/- 1%	19% +/- 6%
PEI <sub>1800</sub>	11% +/- 3%	23% +/- 8%
Biodegradable		
BCP-1	35% +/- 4%	46% +/- 3%
BCP-2	37% +/- 7%	51% +/- 5%

The data in Table 3 show that synthetic VSVG vesicles also enhanced cationic polymer mediated gene transfer efficiencies in COS7 cells with green fluorescent protein reporter gene. Using synthetic VSVG vesicles, the number of GFP positive cells increased by 6 fold as compared to those transfected without using VSVG vesicles.

Table 4: The Effect of Synthetic VSVG Vesicles On Cationic Polymer Mediated FITC-Antisense Oligonucleotide Delivery

Transfection Efficiency (FITC Positive Cells, %)		
In Vitro in COS7 cells		
Polymers	Without Synthetic Vesicle	With Synthetic Vesicle
PLL <sub>25k</sub>	1%+/-0.5%	16%+/-6%
PEI <sub>1800</sub>	7%+/-3%	28%+/-5.5%
BCP-1	23%+/-5%	35%+/-7%
BCP-2	21%+/-5%	32%+/-4%

The data in Table 4 show demonstrate the effects of synthetic VSVG vesicles on cationic polymer (biodegradable and non-biodegradable) mediated GFP reporter gene delivery efficiencies. With the use of synthetic VSVG vesicles, the efficiency of cationic polymer-mediated GFP reporter gene transfer increased by up to 10 fold as compared to the equivalent transfections done without synthetic VSVG vesicles.

10 Table 5. The Effect of VSVG Vesicles On Cationic Polymer Mediated siRNA Delivery

Transfection Efficiency (GFP Positive Cells%)		
In Vitro in 293 cells		
Polymers	Without VSVG Vesicle	With VSVG Vesicle
PLL <sub>25k</sub>	85%+/-7%	65%+/-8%
PEI <sub>1800</sub>	89%+/-6%	67%+/-9%
BCP-3	45%+/-6%	17%+/-9%

The data in Table 5 show the effect of VSVG vesicles on cationic polymer mediated siRNA delivery, evaluated by percentage of GFP positive cells. The vesicles are used to transfect cells that constitutively express GFP protein. The successful delivery of siRNA to a cell results in the inhibition of GFP expression in that cell. In all transfections, the GFP gene expression was inhibited to a degree after the addition of the complex of cationic polymer and siRNA. Lower percentages of GFP positive cells indicates better inhibition of GFP expression, which in turn indicates a higher efficiency in siRNA delivery. The data in

table 8 indicated that VSVG vesicles significantly enhance siRNA delivery compared to delivery of siRNA by cationic polymer alone.

#### EXAMPLE 4

##### 5 Cytotoxicity Assays in HT1080Cells Adding VSVG Vesicle

PEI and polyamidoamine dendrimer were selected for evaluation of the effects of viral envelope vesicles on the reduction of cationic polymer gene carrier cytotoxicity, because PEI and dendrimer were reported to have a high toxicity to the cells both in vitro and in vivo studies. The cytotoxicities of cationic gene carrier on mammalian cells were evaluated using 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) described by Yu et al., (Yu, L., Nielsen, M., and Kim, SW. TerpelxDNA Gene Carrier System Targeting to Artery Wall Cells. J. Controlled Release 72:179-189 (2001)). Briefly,

10 HT1080 cells,  $2 \times 10^4$  cells/well, were seeded in 96-well plates and incubated for 24 hr. Various amounts of polymeric carriers were added to the cells for a period of six hours.

15 The media was then removed and fresh media added. Following further incubation for 48 hrs, the media was removed and 200 $\mu$ l of MTT solution (0.5 mg/ml) was added to each well, and incubated for 3 hrs. The medium was then removed and 200 $\mu$ l DMSO was added to dissolve the formazan crystals. The absorbance of the solution was measured at 570 nm. Cell viabilities was calculated using the equation: Viability (%) = {Abs<sub>570</sub> (sample)}

20 /Abs<sub>570</sub> (control)} x 100. All the experiments were conducted in triplicate. Results obtained with optimal PEI amounts were compared in Table 6. Briefly, HT1080 cells,  $2 \times 10^4$  cells/well, were seeded in 96-well plates and incubated for 24 hr. Various amounts of VSVG vesicles were added to PEI/pDNA complex (500 ng/500 ng) solutions and then were added to the cells for a period of six hours. The remaining steps are the same as those used in the cytotoxicity assay described above. All the experiments were conducted in triplicate. Results obtained with VSVG vesicles and synthetic VSVG vesicles with cationic polymer were showed in Table 7.

Table 6. The Effect of VSVG Vesicles on Reducing Cytotoxicity of PEI and

30 Polyamidoamine Dendrimer

#### Cell Viability

VSVG amount	PEI/DNA (1:1)	Dendrimer/DNA (25:1)
-------------	---------------	----------------------

0	0	0
12.5	35%+/-5%	37%+/-4%
25	44%+/-7%	46%+/-6%
50	65%+/-9%	68%+/-10%
100	74%+/-13%	76%+/-7%
200	81%+/-8%	83%+/-12%
500	85%+/-11%	89%+/-8%

The data in Table 6 show that VSVG vesicles also reduced cationic polymer-induced cytotoxicity and improved cell viabilities. The protective effect of the VSVG vesicles is dose dependent and reaches a saturated state when 500 ng of VSVG protein is used.

Table 7 The Effect of VSVG Vesicles and Synthetic VSVG Vesicles on Reducing Cytotoxicities Of Cationic Polymer/Plasmid DNAComplex and Cationic Polymer/Antisense Oligonucleotide Complex

Cell Viability (MTT Assay)		
In HT1080 Cells		
Polymer-DNA Complexes	Without VSVG Vesicle	With VSVG Vesicle
PLL <sub>25k</sub> -pCMV-GFP	45%+/-4%	92%+/-6%
BCP-1- pCMV-GFP	75%+/-3	97%+/-2
In HT1080 Cells		
Polymer-DNA Complexes	Without Synthetic Vesicle	With Synthetic Vesicle
PLL <sub>25k</sub> -pCMV-GFP	45%+/-4%	89%+/-8%
BCP-1- pCMV-GFP	75%+/-3	91%+/-4%

The data in Table 7 show that both native and synthetic VSVG vesicles protected cells from cationic polymer-induced damage. The percentage of cells that are viable after transfection can double with the use of VSVG vesicles.

EXAMPLE 5Use of Mellitin as a Membrane Active Polypeptide with Lipid-Based Vesicles for Transfection

Mellitin is the major component of bee venom. It is composed of 26 amino acids and forms a cationic peptide that disrupts membranes. In aqueous solution, melittin forms amphipathic  $\alpha$ -helices that interact with lipid membranes via a positively charged cluster (KRKR) near the C terminus, inserting into the lipid bilayer and perturbing the structure. These activities, combined with its net positive charge, make melittin an interesting candidate for enhancing the delivery of DNA in transfection protocols. In these experiments, synthetic mellitin vesicles were prepared in the same manner as the synthetic VSVG vesicles above. The synthetic mellitin vesicles can significantly enhance the efficiency of gene transfection mediated by biodegradable (BCP-3) and non-biodegradable (PLL<sub>25k</sub> and PEI<sub>1800</sub>) cationic polymers by up to 15 fold.

Table 8. The Effective Of Mellitin Peptide Vesicle On Enhancing Cationic Polymer Mediated Gene Delivery.

Transfection Efficiency (GFP Positive Cells%)		
In Vitro in COS7 cells		
Polymers	Without Synthetic Vesicle	With Synthetic Vesicle
PLL <sub>25k</sub>	1.3%+/-0.6%	18%+/-5%
PEI <sub>1800</sub>	5.7%+/-4%	31%+/-4%
BCP-3	23.7%+/-6.5%	41%+/-5%

The data in Table 8 show the effects of the vesicles on the efficiency of transfection. The synthesized mellitin-vesicles significantly enhanced cationic polymer-mediated gene delivery to mammalian cells, up to 14 fold in case of poly-L-Lysine-mediated GFP reporter gene delivery.

WHAT IS CLAIMED IS:

1. A vector for transfecting a eukaryotic cell, comprising a nucleic acid, a nucleic acid binding polymer, a lipid-based vesicle and a membrane active polypeptide.
2. The vector of Claim 1, having a transfection efficiency and cytotoxicity that is significantly improved in relation to a comparable vector comprising said polymer in the absence of said lipid-based vesicle and said membrane active polypeptide.  
5
3. The vector of Claim 34, wherein said the nucleic acid is selected from the group consisting of DNA, RNA, and DNA/RNA hybrid.
4. The vector of Claim 3, wherein said DNA is selected from the group consisting of a linear molecule, a circular molecule, and a single stranded oligodeoxynucleotide.  
10
5. The vector of Claim 4, wherein said circular molecule is plasmid DNA.
6. The vector of Claim 3, wherein said RNA is selected from the group consisting of single stranded RNA and double stranded RNA.  
15
7. The vector of Claim 6, wherein said single stranded RNA is a ribozyme.
8. The vector of Claim 6, wherein said double stranded RNA is a small interference RNA.  
20
9. The vector of Claim 1, wherein said nucleic acid binding polymer has a molecular weight of at least 400 Da.
10. The vector of Claim 1, wherein the molecular structure of said nucleic acid binding polymer is selected from the group consisting of linear, branched, dendrimer and star-shaped.  
25
11. The vector of Claim 1, wherein said nucleic acid binding polymer is a graft copolymer or a block copolymer.
12. The vector of Claim 1, wherein said nucleic acid binding polymer is a biodegradable polymer.  
30
13. The vector of Claim 1, wherein said nucleic acid binding polymer is a non-biodegradable polymer.
14. The vector of Claim 1, wherein said nucleic acid binding polymer is a cationic polymer.
15. The vector of Claim 12, wherein said biodegradable polymer is selected from the group consisting of hydrolysable polymer, pH sensitive cleavable polymer, light  
35

sensitive cleavable polymer, temperature sensitive cleavable polymer, sonication sensitive cleavable polymer, and enzymatically cleavable polymer.

16. The vector of Claim 14, wherein said cationic polymer is selected from the group consisting of poly-L-lysine, polyethelenimine, poly[a-(-aminoutyl)-L-glycolic acid],  
5 chitosan, polyamidoamine, and poly(2-dimethylamino)ethyl methacrylate.

17. The vector of Claim 9, wherein said dendrimer has more than three branches.

18. The vector of Claim 14, wherein said cationic polymer and said nucleic acid have a ratio of about 1:1 to 50:1

10 19. The vector of Claim 1, wherein said lipid-based vesicle is composed of material selected from the group consisting of a mammalian cell membrane and a lipid mixture.

20. The vector of Claim 19, wherein said lipid mixture comprises, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine.

15 21. The vector of Claim 20, wherein said lipid mixture comprising phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine are in a ratio of about 6:2:2 by weight, respectively.

22. The vector of Claim 1, wherein said membrane active polypeptide is a viral protein.

20 23. The vector of Claim 1, wherein said membrane active polypeptide is a non-viral protein.

24. The vector of Claim 22, wherein said viral protein is selected from the group consisting of a wild-type envelope protein and a recombinant envelope protein.

25 25. The vector of Claim 22, wherein said viral protein is a vesicular stomatitis virus glycoprotein.

26. The vector of Claim 22, wherein said viral protein comprises a monomer of vesicular stomatitis virus glycoprotein.

30 27. The vector of Claim 25, wherein said vesicular stomatitis virus glycoprotein is selected from the group consisting of a wild type vesicular stomatitis virus glycoprotein mature protein, a wild type vesicular stomatitis virus glycoprotein peptide and a recombinant vesicular stomatitis virus glycoprotein polypeptide.

28. The vector of Claim 1, wherein said polymer and said nucleic acid are in the form of a complex.

29. The vector of Claim 28, wherein said complex is contained by said membrane active polypeptide and lipid-based vesicle.

5 30. The vector of Claim 28, wherein said complex is in contact with said membrane active polypeptide and lipid-based vesicle

10 31. A method of making a vector according to Claim 1 comprising:  
isolating a nucleic acid;  
combining said nucleic acid with a nucleic acid binding polymer to form a complex;

providing a plurality of lipid-based vesicles, said lipid-based vesicles comprising at least one membrane active polypeptide; and  
combining said complex with said plurality of lipid-based vesicles.

15 32. The method of Claim 31, wherein said nucleic acid binding polymer is a cationic polymer.

33. The method of Claim 32, wherein the cationic polymer is selected from the group consisting of poly-L-lysine, polyethelenimine, poly[a-(aminoutyl)-L-glycolic acid], chitosan, polyamidoamine, and poly(2-dimethylamino)ethyl methacrylate.

34. The method of Claim 31, wherein said polymer is a biodegradable polymer.

20 35. The method of Claim 34, wherein said biodegradable polymer is selected from the group of consisting of hydrolysable polymer and pH sensitive cleavable polymer.

36. The method of Claim 35, wherein said hydrolysable polymer is biodegradable cationic polymer.

25 37. The method of Claim 35, wherein said pH sensitive cleavable polymer is a polyacetal polymer.

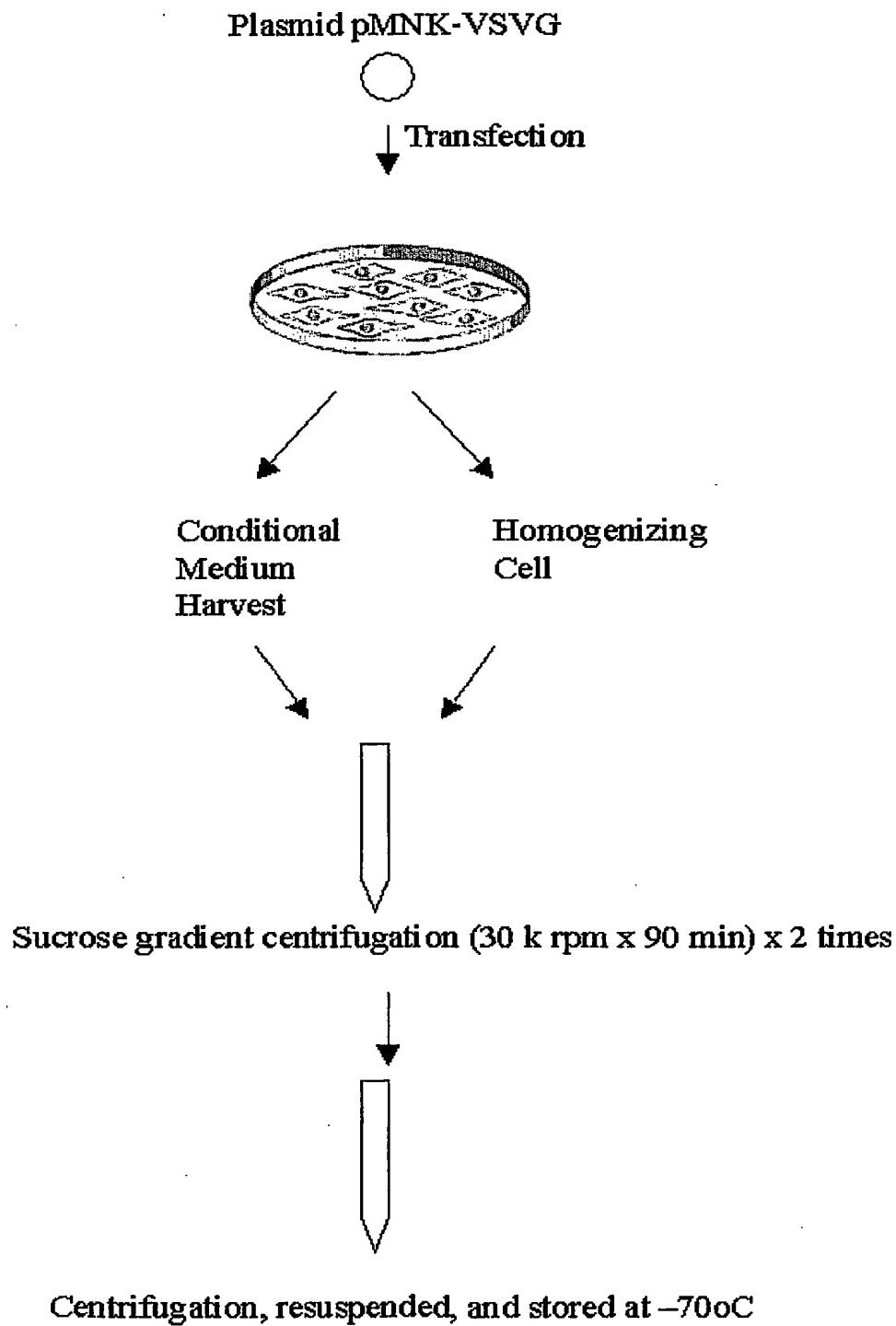
38. The method of Claim 31, wherein said vesicles comprise a native lipid membrane.

39. The method of Claim 31, wherein said vesicles comprise a synthetic lipid membrane.

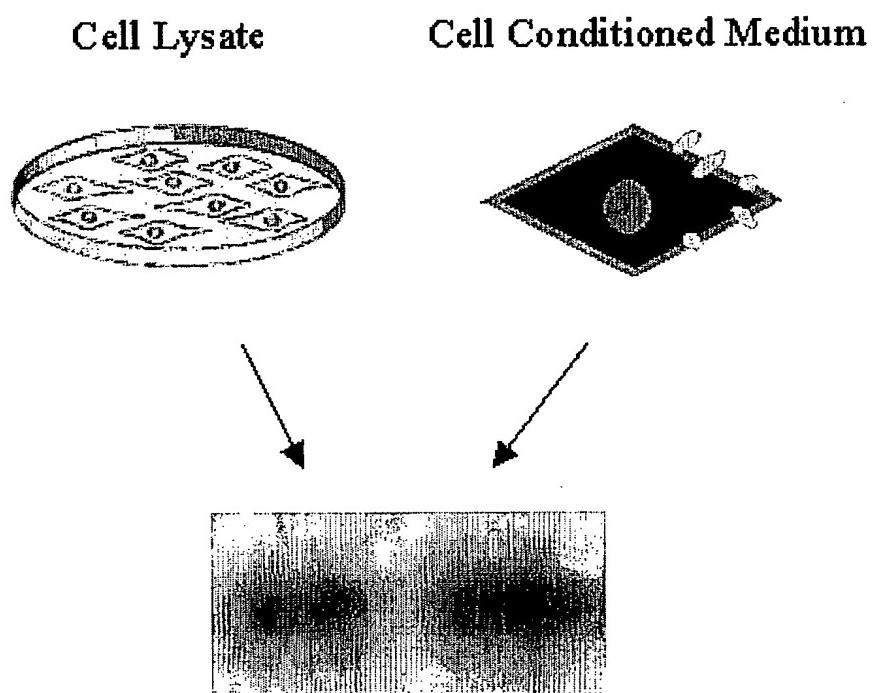
30 40. The method of Claim 31, wherein said membrane active polypeptide is selected from the group consisting of vesicular stomatitis virus glycoprotein or a portion of VSVG that is membrane active.

41. A method of gene therapy comprising:
  - identifying an individual in need of gene therapy; and
  - administering the vector of Claim 1 to said individual in a therapeutically effective amount.
- 5 42. The method of Claim 41, wherein said individual is a mammal.
43. A method of introducing a nucleic acid into a cell comprising contacting said cell with the vector of Claim 1.
- 10 44. The method of Claim 43, wherein said cell is a eukaryotic cell selected from the group consisting of a human fibroblast, an animal embryo stem cell, a keratinocyte, a pancreatic cell, a myocardium cell, a bone marrow cell, a neuronal cell, and a macrophage.

**Figure 1: VSVG Vesicle Preparation**



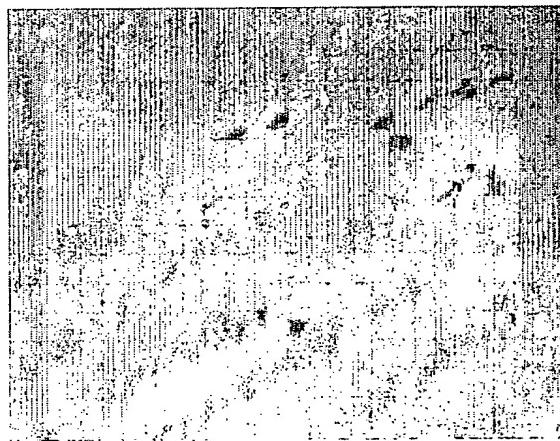
**Figure 2 Western Blot for Detection of VSVG protein**



**Figure 3: The Effects of VSVG on PLL-mediated Gene Transfer  
In Ht-1080 Cells and HepG2 Cells**

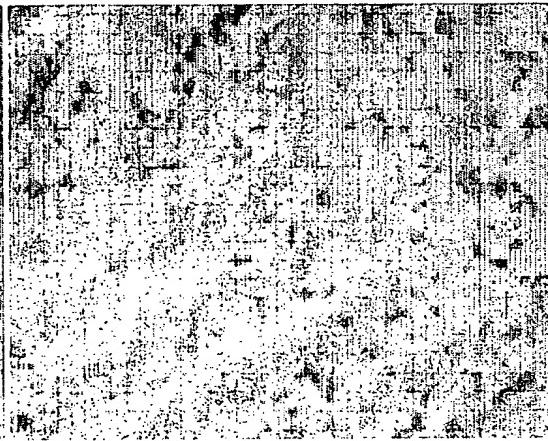
**FIG. 3A**

PLL-pDNA

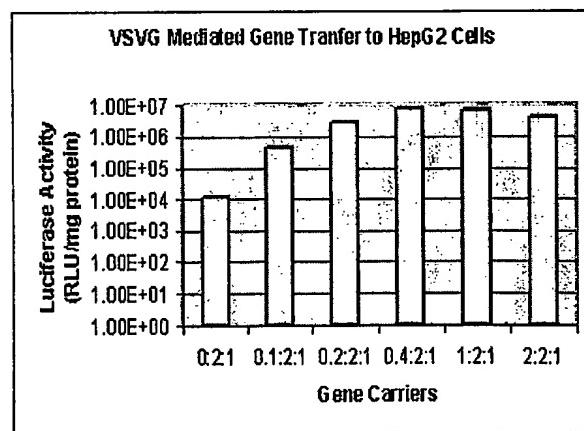


**FIG. 3B**

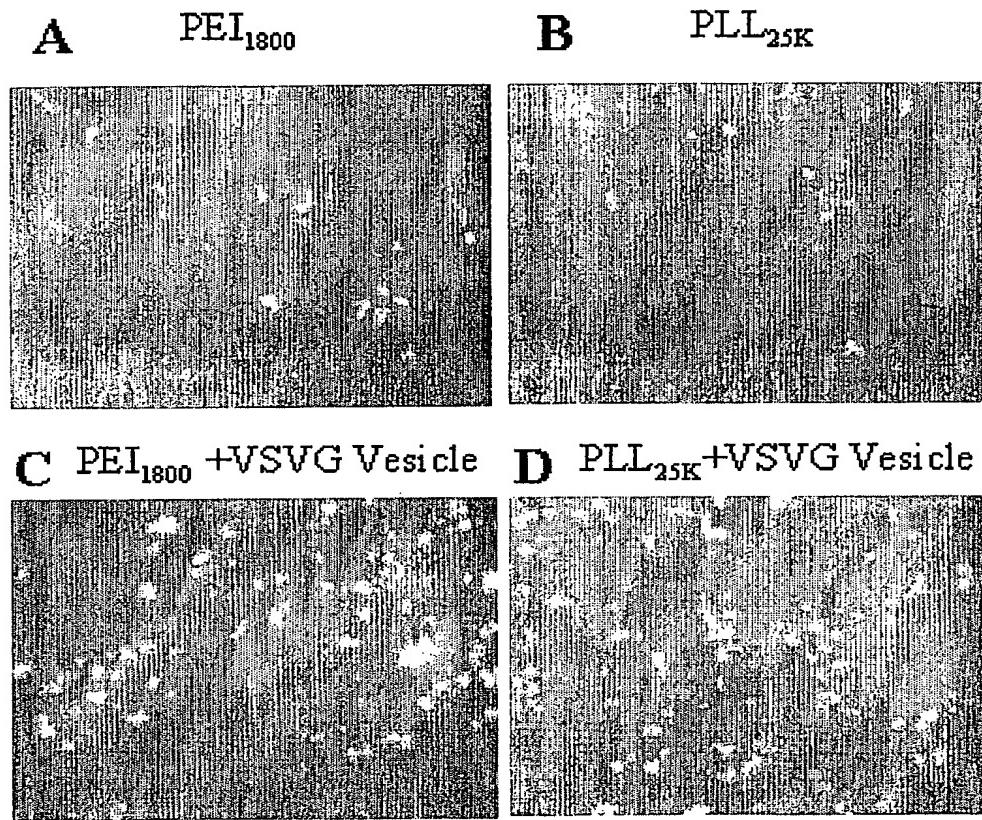
VSVG-PLL-pDNA

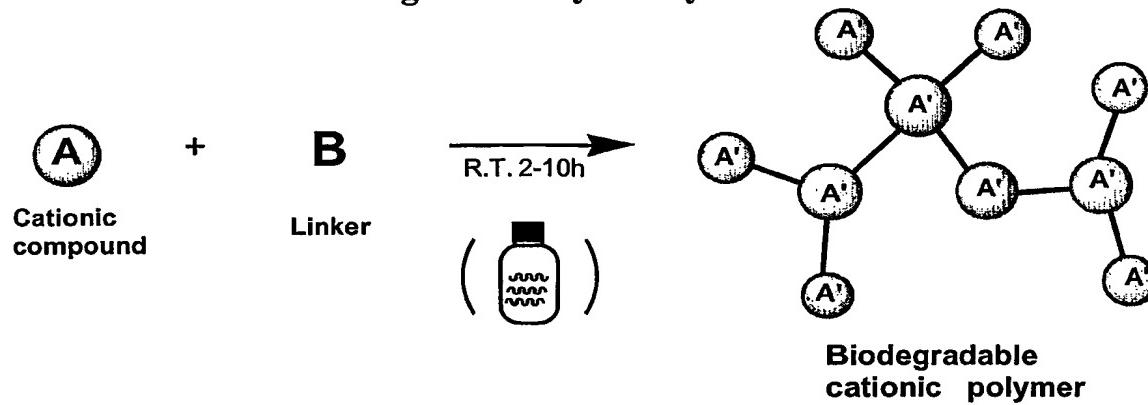


**FIG. 3C**



**Figure 4 The Effect of VSVG Vesicles On Cationic Polymer Mediated Antisense Oligonucleotide Delivery**



**Figure 5: Polymer Synthesis**

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C12N 15/00, C07H 21/02, C07K 1/00

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(71) Applicant (for all designated States except US): **NITTO DENKO CORPORATION [JP/JP]; 1-1-2, Shimohozumi, Ibaraki-shi, Osaka, 567-8680 (JP).**

Declaration under Rule 4.17:

— of inventorship (Rule 4.17(iv)) for US only

(72) Inventors; and

(75) Inventors/Applicants (for US only): **YU, Lei [CA/US]; 3682 Strata Drive, Carlsbad, CA 92008 (US). MATSUMOTO, Kenji [JP/US]; 18181 Sun Maiden Court, San Diego, CA 92127 (US).**

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: VECTOR FOR TRANSFECTION OF EUKARYOTIC CELLS

(57) Abstract: Vectors comprising a nucleic acid, a nucleic acid binding polymer, a vesicle and a membrane active polypeptide are described. Preferred vectors facilitate transfection and/or reduce cytotoxicity. Methods of making the vectors and methods of using the vectors to transfect cells and/or treat a patient in need of gene therapy are described.

**INTERNATIONAL SEARCH REPORT**

International application No. PCT/US03/07426	
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**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : A61K 9/127; C12N 15/00; C07H 21/02; C07K 1/00  
US CL : 424/450; 435/320.1; 536/23.1; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
U.S. : 424/450; 435/320.1; 536/23.1; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
STN/WEST

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TRUBETSKOY et al, Cationic liposomes enhance targeted delivery and expression of exogenous DNA mediated by N-terminal modified poly(L-lysine)-antibody conjugate in mouse lung endothelial cells, <i>Biochim. Biophys. Acta</i> 1992, Vol. 1131, pages 311-313.	1-5, 9, 10, 12, 14-16, 18, 19, 23, 28, 30
—		————
Y		6-8, 20-22, 24-27
A	TRUBETSKOY et al, Use of N-Terminal Modified Poly(L-lysine)-Antibody Conjugate as a Carrier for Targeted Gene Delivery in Mouse Lung Endothelial Cells, <i>Bioconjug. Chem</i> 1992, Vol 3, pages 3232-327.	1-5, 9, 10, 12, 14-16, 18, 19, 23, 28, 30
X	US 5908777 A (LEE et al) 01 June 1999, see Figs 1 and 4; column 1, lines 5-8; column 3, lines 21-27; column 8, lines 22-29; column 5, lines 48-51; column 7, lines 22-27 and 41-42; column 5, lines 15-22	1-5, 9, 10, 12, 14-16, 19, 22, 23, 28-30
—		————
Y	OKIMOTO et al, VSV-G Envelope Glycoprotein Forms Complexes with Plasmid DNA and MLV Retrovirus-like Particles in Cell-free Conditions and Enhances DNA Transfection, <i>Mol. Ther.</i> , Vol. 4, No. 3, September 2001, pages 232-238, see entire document.	6-8, 20, 21, 24-27 20-28, 30

Further documents are listed in the continuation of Box C.

See patent family annex.

• Special categories of cited documents:		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"B"	earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

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*Janice Ford*  
*for*

PCT/US03/07426

## INTERNATIONAL SEARCH REPORT

## C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5753258 A (SCHREIER et al) 19 May 1998, see column 3, lines 40-61; and column 7, lines 47-60.	20-27
Y	US 5472852 A (SMIRNOV et al) 05 December 1995, see column 3, lines 56-64; column 4, lines 3 and 4; and column 10, lines 40-43.	21

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US03/07426

**Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claim Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claim Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claim Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:  
Please See Continuation Sheet

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-10,12,14-16 and 18-30, species = polylysine

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

PCT/US03/07426

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING**

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group 1, claim(s) 1-30, drawn to a vector comprising a nucleic acid, a nucleic acid binding polymer, a lipid vesicle, and a membrane active polypeptide.

Group 2, claim(s) 31-40, drawn to methods of making a vector comprising a nucleic acid, a nucleic acid binding polymer, a lipid vesicle, and a membrane active polypeptide.

Group 3, claim(s) 41-44, drawn to methods of gene therapy comprising administering to a patient a vector comprising a nucleic acid, a nucleic acid binding polymer, a lipid vesicle, and a membrane active polypeptide.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

nucleic acid binding polymers that may be linear, branched, dendrimer, and star-shaped, graft copolymers, block copolymers, non-biodegradable, biodegradable selected from the group consisting of hydrolysable polymer, pH sensitive cleavable polymer, light sensitive cleavable polymer, temperature sensitive cleavable polymer, sonication sensitive cleavable polymer, and enzymatically cleavable polymer, or that may be selected from the group consisting of poly-L-lysine, polyethylenimine, poly[ $\alpha$ -( $\omega$ -aminobutyl)-L-glycolic acid], chitosan, polyamidoamine, and poly(2-dimethylamino)ethyl methacrylate.

The inventions listed as Groups 1-3 do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The technical feature linking the claimed inventions is a vector comprising a nucleic acid, a nucleic acid binding polymer, a lipid vesicle, and a membrane active polypeptide. However, the prior art anticipates this vector, so the technical feature linking the inventions cannot be a special technical feature under PCT Rule 13.2. See for example, Lee et al (US Patent 5,908,777, issued 6/1/99). Lee taught a liposome-based vector comprising a nucleic acid, poly-lysine, and a membrane active polypeptide. See claim 3, wherein the fusogenic peptide or receptor-specific ligand is considered to be a membrane active polypeptide.

The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: As noted above, at least one species of the invention was known in the prior art (Lee et al 1999), so there is no special technical feature linking the various species recited in the claims.

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The first recited species is linear, biodegradable, polylysine, readable on claims 1-10, 12, 14-16, and 18-30.